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Published in:
European Journal of Biochemistry

DOI:
10.1046/j.1432-1327.2000.00993.x

Publication date:
2000

Eur. J. Biochem. **267**, 228–234 (2000) © FEBS 2000

A spectroscopic assay for the analysis of carbohydrate transport reactions

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A carbohydrate-transport assay was developed that does not require isotopically labelled substrates, but allows transport reactions to be followed spectrophotometrically. It makes use of a membrane system (hybrid membranes or proteoliposomes) bearing the transport system of interest, and a pyrroloquinoline quinone-dependent aldose dehydrogenase [soluble glucose dehydrogenase (sGDH)] and the electron acceptor 2,6-dichloroindophenol (Cl_2Ind) enclosed in the vesicle lumen. After transport across the vesicular membrane, the sugar is oxidized by sGDH. The accompanying reduction of Cl_2Ind results in a decrease in A_{600}. The assay was developed and optimized for the lactose carrier (LacS) of *Streptococcus thermophilus*, and both solute/H^+ symport and exchange types of transport could be measured with high sensitivity in crude membranes as well as in proteoliposomes. To observe exchange transport, the membranes were preloaded with a nonoxidizable substrate analogue and diluted in assay buffer containing an oxidizable sugar. Transport rates measured with this assay are comparable with those obtained with the conventional assay using isotopically labelled substrates. The method is particularly suited for determining transport reactions that are not coupled to any form of metabolic energy such as uniport reactions, or for characterizing mutant proteins with a defective energy-coupling mechanism or systems with high-affinity constants for sugars.

Keywords: aldose dehydrogenase; carbohydrate; kinetics; spectroscopic assay; transport.

Bacteria have evolved different transport systems to fulfil their requirement to take up nutrients or to release unwanted products. Basically, three distinct transport mechanisms exist for carbohydrates: (a) primary transport systems, which couple the uptake of sugar to the hydrolysis of ATP; (b) secondary transport systems, which couple the uptake of sugar to the downhill transport of another solute (e.g. H^+ or Na^+ ions) in the same direction (symport), to the subsequent metabolism of the sugar (uniport) or to the downhill transport of another solute in the opposite direction (antiport or exchange); (c) phospho*enol*pyruvate-dependent phosphotransferase systems (phospho*enol*pyruvate-PTS), which catalyze uptake concomitant with phosphorylation of the sugar.

Translocation of sugars via any of these transport mechanisms is usually analyzed by following the distribution of isotopically labelled substrates across the membrane, i.e. between the outer and inner compartment of a membrane system. As not all substrates are available in an isotopically labelled form, and conventional transport assays have a number of limitations, we devized an alternative transport assay method that is applicable to the study of primary and secondary carbohydrate-transport systems, but may also even find a use in the study of phospho*enol*pyruvate–PTSs as discussed below.

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Abbreviations: Cl_2Ind, 2,6-dichloroindophenol; sGDH, soluble quinoprotein glucose dehydrogenase; α-NPG, α-naphthyl-α-D-galactopyranoside; TMG, methyl-β-D-thiogalactoside; PTS, phospho*enol*pyruvate–sugar phosphotransferase system; PQQ, pyrroloquinoline quinone.

Enzyme: glucose dehydrogenase (EC 1.1.99.17).

(Received 17 September 1999, accepted 4 November 1999)

The idea for the assay was born out of our research on a putative xyloside-transport system, XylP, which is homologous to the lactose transporter (LacS) of *Streptococcus thermophilus* studied here. To demonstrate the usefulness of the method, it was necessary to first apply it to the well-characterized LacS protein, as virtually nothing is known about the XylP system.

The new assay requires a membrane system in which a carbohydrate-transport protein is present in a liposome or membrane vesicle, and in which the soluble pyrroloquinoline quinone (PQQ)-dependent aldose dehydrogenase (sGDH) is enclosed. sGDH, originating from *Acinetobacter calcoaceticus*, is a homodimeric enzyme containing one PQQ and two Ca^{2+} ions per monomer [1–5]. The enzyme has a broad substrate specificity and a high turnover. On the condition that the substrate has a hydroxy group at the C-1 position, sGDH catalyzes not only the oxidation of monosaccharides such as glucose, xylose and galactose, but also disaccharides such as lactose and melibiose. Oligosaccharides may also be substrates of the enzyme, albeit with a lower affinity than monosaccharides or disaccharides [6]. On oxidation of the aldose sugar to the corresponding aldono-δ-lactone, the PQQ cofactor is reduced and subsequently reoxidized by artificial dyes such as 2,6-dichloroindophenol (Cl_2Ind) and phenazine methosulfate [3,6,7]. Cl_2Ind has a high extinction coefficient with an absorption maximum at 600 nm, which allows its reduction to be followed spectrophotometrically with high sensitivity, even in the presence of membranes. The apo form (without the PQQ factor) of sGDH can easily be isolated from a recombinant *Escherichia coli* strain, carrying the gene for sGDH, and the enzyme is readily reconstituted with PQQ *in vitro* [8,9].

Here, we report the use of sGDH as a coupling enzyme for monitoring carbohydrate-transport reactions. The assay is used to follow the activity of the LacS protein, which catalyzes the uptake of a variety of galactosides either in symport with a proton or in exchange for another sugar. The former reaction is

driven by the proton motive force, whereas in the exchange reaction two substrates are transported in opposite directions without net movement of a proton [10,11]. Examples of oxidizable substrates of LacS are lactose, galactose and melibiose, irrespective of whether they are in the α-anomeric or β-anomeric form. Methyl-β-D-thiogalactoside (TMG) is a nonoxidizable lactose analogue, and α-naphthyl-α-D-galactopyranoside (α-NPG) is a nonoxidizable high-affinity competitive inhibitor of LacS [12,13].

EXPERIMENTAL PROCEDURES

Materials

D-[*glucose*-1-^{14}C]Lactose (2.11 TBq·mol^{-1}) was obtained from The Radiochemical Centre, Amersham, Bucks., UK. The sGDH and PQQ were generously provided by Professor J. A. Duine from the Technical University of Delft, the Netherlands. *n*-Dodecyl-β-D-maltoside was obtained from Sigma and Triton X-100 from Boehringer-Mannheim. The 0.45-μm cellulose nitrate filters were from Schleicher & Schuell GmbH, Dassel, Germany. Total *E. coli* lipids were obtained from Avanti Polar Lipids, and L-α-phosphatidylcholine from egg yolk was from Sigma. All other materials were reagent grade and obtained from commercial sources.

Bacterial strains and growth conditions

S. thermophilus ST11 (Δ*lacS*) carrying plasmid pGKHis was grown semianaerobically at 42 °C in Eliker broth supplemented with 0.5% beef extract, 20 mM lactose and 5 μg·mL^{-1} erythromycin [14]; pGKHis bears the *lacS* gene.

Isolation of membrane vesicles and membrane fusion

Right-side-out membrane vesicles of *S. thermophilus* were isolated as described previously [15] with the following modifications: the cell wall was digested with 10 mg·mL^{-1} lysozyme, and DNase and RNase were added to final concentrations of 100 μg·mL^{-1} each. For transport studies, peripheral membrane proteins as well as cytosolic contaminants were removed by extracting the membrane vesicles with 5 M urea and 6% (w/w) sodium cholate as described [16]. Membranes treated with urea and sodium cholate contain ≈ 25% LacS on a protein basis. These membrane vesicles were fused with liposomes to obtain a more tightly sealed membrane preparation [11]. For the fusion, the membrane vesicles were mixed with liposomes in a 1 : 10 ratio (w/w) and stored in liquid nitrogen with or without sGDH (see below), in portions of 400 μL containing 0.8 mg total protein and 8 mg lipid. Before use, the membranes were thawed at room temperature and extruded 11 times through 400-nm polycarbonate filters; the preparations obtained are termed hybrid membranes. Liposomes were prepared from acetone/ether-washed *E. coli* lipids and L-α-phosphatidylcholine from egg yolk in a ratio of 3 : 1 (w/w) and dissolved in 50 mM potassium phosphate, pH 7.0, to a final phospholipid concentration of 20 mg·mL^{-1}. The protein concentration of the membranes was determined with the Bio-Rad DC Protein Assay according to the manufacturer's instructions (Bio-Rad).

Purification and reconstitution of LacS

The LacS protein was purified and reconstituted essentially as described [14,17]. Protein purified in *n*-dodecyl-β-D-maltoside was reconstituted into Triton X-100-destabilized liposomes to a final lipid to protein ratio of 40 : 1 (w/w). The proteoliposomes were stored in liquid nitrogen with or without sGDH (see below). The concentration of purified LacS was determined by measurement of A_{280} (ε_{280} = 76 320 M^{-1}·cm^{-1}).

sGDH reconstitution and activity assay

sGDH was prepared as described [9], using a 1.5-fold excess of PQQ over apo-sGDH. Briefly, sGDH and PQQ were mixed at final concentrations of 25 μM and 37.5 μM, respectively, in 20 mM Mops, pH 7.0, plus 3 mM $CaCl_2$. The mixture was incubated for 15 min at room temperature and, subsequently, used in the enzymatic assays or for enclosure in the membranes. Enzyme assays were performed in 50 mM potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and various concentrations of substrate. The reactions were started by the addition of 78 ng·mL^{-1} sGDH (enzyme samples were diluted with 20 mM Mops buffer, pH 7.0, containing 3 mM $CaCl_2$ and 10% glycerol), and the enzyme activity was detected spectrophotometrically at 25°C by following the reduction of Cl_2Ind at 600 nm [9].

Enclosure of sGDH in proteoliposomes or hybrid membranes

For the enclosure of sGDH, proteoliposomes or hybrid membranes (20 mg·mL^{-1} phospholipid) were mixed with various concentrations sGDH in 50 mM potassium phosphate, pH 7.0, supplemented with 1 mM $MgSO_4$ in a total volume of 400 μL. The sGDH concentrations used for the enclosure procedures are specified in the figure legends. After freezing in liquid nitrogen and slow thawing at room temperature, the mixture was extruded 11 times through a 400-nm polycarbonate filter [18]. To remove external sGDH, the proteoliposomes or hybrid membranes were washed four times with 50 mM potassium phosphate, pH 7.0, by centrifugation (15 min, 185 000 ***g***, 10°C). The proteoliposomes and hybrid membranes were resuspended to a final concentration of 3 mg·mL^{-1} LacS, which corresponds to 120 mg·mL^{-1} total phospholipid. When proteoliposomes or hybrid membranes were used for so-called exchange-type or counterflow-type experiments, the enclosure of sGDH and the washing steps were performed in the presence of 5 mM TMG.

Spectrophotometric measurements of LacS transport activity

Transport of sugars was determined from the decrease in A_{600} of Cl_2Ind at a temperature of 25°C (ε_{600} = 20.6 mM^{-1}·cm^{-1}). The reaction mixture consisted of 50 mM potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and various concentrations of sugar. The reaction was started by the addition of sGDH-containing proteoliposomes or hybrid membranes to a final concentration of 30 μg·mL^{-1} LacS, which corresponds to 1.2 mg·mL^{-1} phospholipid (5 μL proteoliposomes or hybrid membranes were added to 500 μL assay buffer).

Measurement of LacS transport activity using radiolabelled lactose

Proteoliposomes were prepared as described previously [14], except that a final lipid to protein ratio of 40 (w/w) was used and 5 mM TMG was enclosed in the vesicle lumen. The proteoliposomes were concentrated by centrifugation (15 min, 185 000 ***g***, 10°C), and resuspended in 50 mM potassium phosphate, pH 7.0, plus 5 mM TMG to a final protein

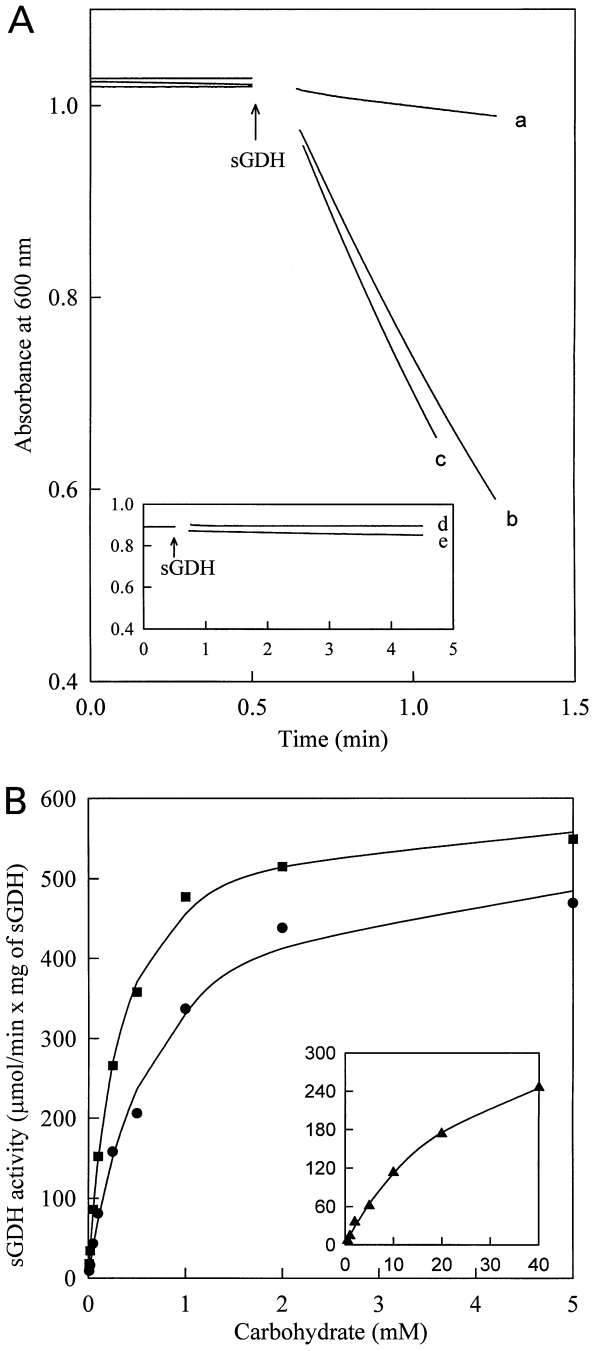

Fig. 1. Kinetics of sugar oxidation by sGDH. (A) Time-dependence of sugar oxidation by sGDH. The assays were performed in 500 μL potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and the following sugars (2 mM): (a) melibiose; (b) lactose; (c) glucose. The inset shows that α-NPG (d) and TMG (e) are not oxidized by sGDH. The arrows indicate the addition of sGDH to a final concentration of 78 ng·mL^{-1}. (B) Concentration-dependence of the initial rate of carbohydrate oxidation: (●) lactose; (■) glucose; (▲) melibiose (the latter is shown in the inset). The data were fitted to the Michaelis–Menten equation.

concentration of about 3 mg·mL^{-1}. The translocation reaction was started by the addition of 2 μL proteoliposomes to 200 μL 50 mM potassium phosphate, pH 7.0, plus 50 μM [^{14}C]lactose at a temperature of 25°C. The reaction was stopped at different time points by diluting the proteoliposomes into 2 mL ice-cold 0.1 M LiCl and rapid filtration over 0.45-μm cellulose nitrate filters. The filters were subsequently washed with another 2 mL ice-cold LiCl, and the radioactivity was counted by liquid-scintillation spectrometry.

RESULTS

Kinetic parameters and substrate specificity of sGDH

sGDH is able to oxidize different carbohydrates with a hydroxy group at the C-1 position (Fig. 1). The carbohydrates used in this study were lactose, glucose, melibiose, TMG, α-methylglucose and α-NPG. At a concentration of 2 mM, the rates of oxidation of glucose and lactose do not differ very much, whereas that of melibiose is much lower (Fig. 1A). Sugars in which the anomeric carbon is methylated, such as TMG and α-methylglucose (not shown), or linked to a naphthyl group, such as α-NPG, are not substrates of sGDH (Fig. 1A; inset). The apparent K_m and V_{max} of sGDH for lactose, glucose and melibiose are 660 μM and 548 μmol·mg^{-1}·min^{-1}, 300 μM and 590 μmol·mg^{-1}·min^{-1} and 27 mM and 410 μmol·mg^{-1}·min^{-1}, respectively (Fig. 1B).

Spectroscopic measurement of LacS activity in proteoliposomes

Proteoliposomes with sGDH present in the vesicle lumen were used to follow the transport of lactose. The transport was assayed in a reaction mixture consisting of 500 μL 50 mM potassium phosphate, pH 7.0, plus 50 μM Cl_2Ind (electron acceptor) and 10 mM lactose. Cl_2Ind is highly membrane permeant and it was not necessary to preload the proteoliposomes with this compound. On addition of 5 μL proteoliposomes to the reaction mixture, A_{600} decreased immediately (Fig. 2A). To demonstrate that this decrease was caused by the facilitated transport of lactose, sGDH-containing liposomes were used in control experiments. Some oxidation of lactose was observed in these liposomes (Fig. 2B), but the decrease in absorbance could be ascribed to sGDH associated with the outer surface of the liposomes by using glucose instead of lactose. Glucose is a substrate of sGDH but the LacS protein does not transport it [13]. The decrease in A_{600} with glucose is thus a measure of the amount of sGDH present at the outer surface of the membranes (Fig. 2A,B). Moreover, in proteoliposomes, the oxidation of lactose could be inhibited by a threefold excess of the high-affinity galactoside α-NPG, whereas it had no effect on the oxidation rate of glucose (data not shown).

On the basis of a specific internal volume of the proteoliposomes of 1 μL·mg^{-1} phospholipid and the specific activity of sGDH, one can calculate the efficiency of trapping of the enzyme in the proteoliposomes. We observed that, in general, 5–10-fold more activity was entrapped than expected when the enzyme would simply equilibrate in the vesicle lumen. The higher internal sGDH concentration probably reflects the binding of the enzyme to the (proteo)liposome membrane, which is consistent with the observation that some residual sGDH is present externally even after extensive washing of the proteoliposomes. This internal binding of sGDH is obviously advantageous for the assay, as it allows the enzyme to be concentrated in the proteoliposomes or hybrid membranes.

Close inspection of the time-dependent decrease in A_{600} reveals that the kinetics of lactose oxidation in the proteoliposomes has multiple components, i.e. an initial slow phase followed by a more rapid oxidation (Fig. 2A, dashed line to curve a). This apparent acceleration of sugar oxidation is not

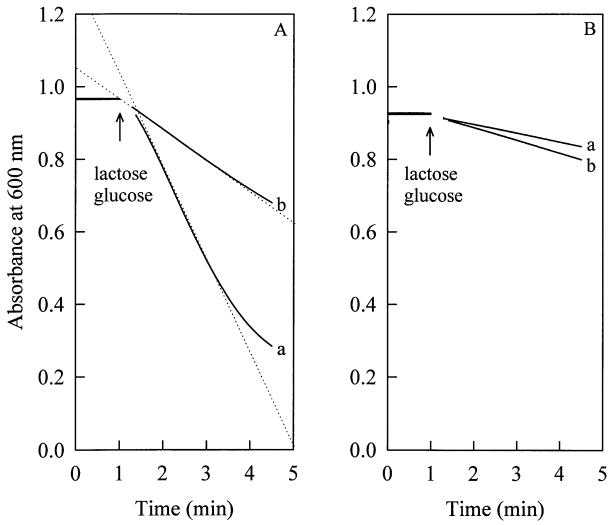

Fig. 2. Oxidation of sugars by sGDH-containing proteoliposomes and liposomes. The sGDH concentration used in the enclosure procedure was 4.5 μg·mL^{-1}. The assays were performed in 300 μL potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and 5 μL proteoliposomes (final concentration: 55.5 μg·mL^{-1} LacS, which corresponds to 2.2 mg·mL^{-1} lipid) (A) or 5 μL liposomes (final concentration 2.2 mg·mL^{-1} lipid) (B). The arrows indicate the addition of 10 mM lactose (a) or glucose (b).

observed in the liposomes with glucose or lactose as substrate nor in the proteoliposomes with glucose as substrate. The change in the rate of oxidation can be explained by the different modes of transport of the LacS protein. LacS not only facilitates a H^+-linked symport reaction, but also an exchange reaction in which two molecules are transported in opposite directions. The homologous exchange of lactose or the heterologous lactose/galactose exchange is an order of a magnitude faster than the lactose/H^+ symport reaction. In the experiment described above, lactose enters the proteoliposome lumen and becomes oxidized. There will thus be a build-up of oxidized lactose (lactono-δ-lactone), which is probably a substrate for the LacS protein and therefore accelerates the reaction by serving as a counter substrate. In other words, the reaction changes from downhill lactose uptake initially to lactose/lactono-δ-lactone exchange at later times.

Transport activity with and without entrapped TMG

To investigate whether or not the presence of a counter substrate accelerates the transport reaction, the proteoliposomes were preloaded with 5 mM TMG. Oxidation of lactose was indeed much faster when TMG-loaded proteoliposomes were used instead of unloaded proteoliposomes (Fig. 3). The higher oxidation rate was not due to a higher sGDH concentration on the outside or the inside of the proteoliposomes, as the activities for loaded and unloaded proteoliposomes were similar on solubilization with 0.08% Triton X-100 (measure for the total sGDH activity present in the assay mixture). Furthermore, the rate of oxidation was highest at the start of the experiment and acceleration was no longer observed. As expected, loading of the (proteo)liposomes had no effect on the oxidation of glucose (data not shown). To demonstrate that the presence of a methylated hexose in the proteoliposome lumen did not

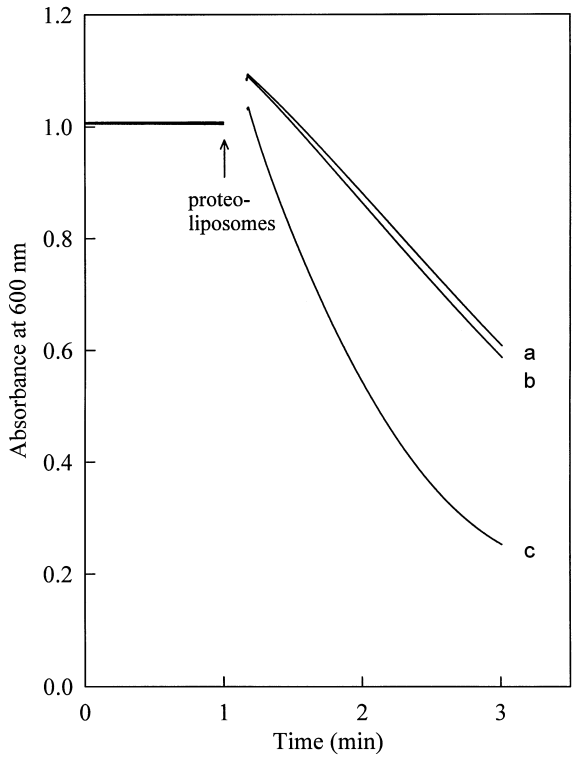

Fig. 3. Transport activity in sGDH-containing LacS proteoliposomes with and without entrapped TMG. The sGDH concentration used in the enclosure procedure was 25 μg·mL^{-1}. The assays were performed in 500 μL potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and 5 mM lactose. The arrow indicates the addition of 5 μL proteoliposomes (final concentration 30 μg·mL^{-1} LacS, which corresponds to 1.2 mg·mL^{-1} lipid) without sugar (a) or preloaded with 5 mM α-methylglucose (b) or 5 mM TMG (c).

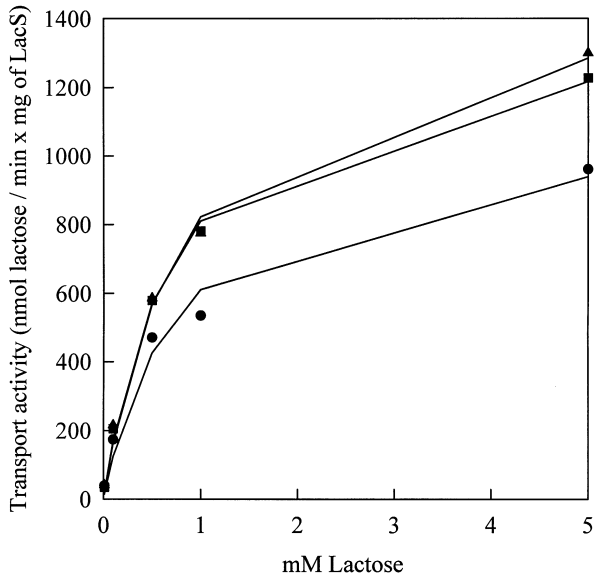

Fig. 4. Kinetics of lactose uptake by proteoliposomes as function of the internal sGDH concentration. The assays were performed in 500 μL potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and various concentrations of lactose. The reactions were started by the addition of 5 μL proteoliposomes (final concentration 30 μg·mL^{-1} LacS, which corresponds to 1.2 mg·mL^{-1} lipid), preloaded with 5 mM TMG and various concentrations of sGDH: (●) 25 μg·mL^{-1}; (■) 50 μg·mL^{-1}; (▲) 75 μg·mL^{-1}. The data were fitted to the Michaelis–Menten equation.

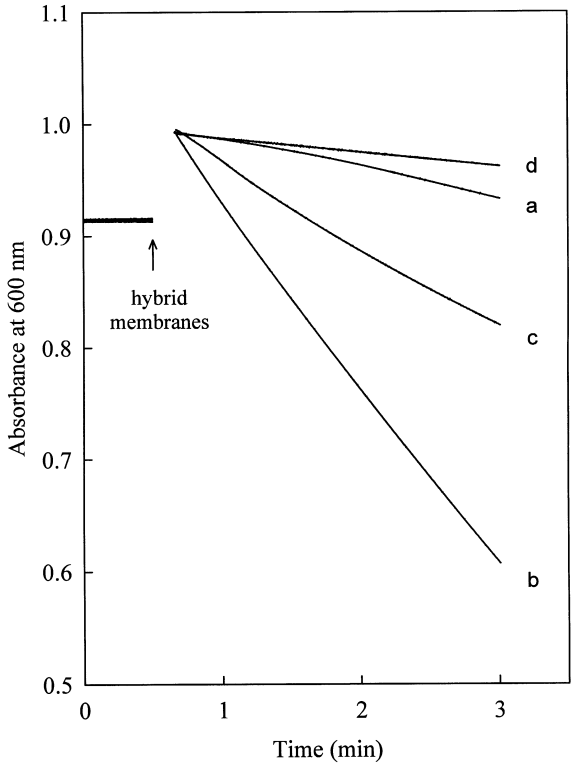

Fig. 5. Transport activity by sGDH-containing hybrid membranes of *S. thermophilus* ST11ΔlacS/pGKHis. The sGDH concentration used in the enclosure procedure was 25 $\mu g \cdot mL^{-1}$. For these studies both unloaded (a) and TMG-loaded (b, c and d) membranes were used. The assays were performed in 500 μL potassium phosphate, pH 7.0, supplemented with 50 μM Cl_2Ind and various concentrations of melibiose in the absence (a, b and c) or presence (d) of a 10-fold excess of TMG. The arrow indicates the addition of 5 μL hybrid membranes (final concentration 30 $\mu g \cdot mL^{-1}$ LacS, which corresponds to 1.2 $mg \cdot mL^{-1}$ lipid). (a) 5 mM melibiose and unloaded membranes; (b) 5 mM melibiose and TMG-loaded membranes; (c) 0.5 mM melibiose and TMG-loaded membranes; (d) 0.5 mM melibiose plus 5 mM TMG and TMG-loaded membranes.

influence the activity of sGDH, proteoliposomes were loaded with α-methylglucose instead of TMG. α-Methylglucose is not a substrate for either sGDH or the LacS protein. Any differences from unloaded proteoliposomes can be attributed to an effect of α-methylglucose on sGDH activity. As the activities of unloaded and α-methylglucose-loaded proteoliposomes were identical, any influence on sGDH could be ruled out. Taken together, the data indicate that the acceleration of lactose oxidation in proteoliposomes reflects an increased contribution in time of lactose uptake via lactose/lactono-δ-lactone exchange.

Transport activity as function of sGDH concentration

To exclude the possibility that the internal sGDH concentration was limiting the transport reaction, different concentrations of sGDH were enclosed. These proteoliposomes were added to assay buffer containing various concentrations of lactose or glucose. The data clearly indicate that the rate of the oxidation reaction was limited by the transport of lactose when the sGDH concentration was ≥ 50 $\mu g \cdot mL^{-1}$ during the enclosure procedure (Fig. 4). At a saturating sGDH concentration, the apparent K_m and V_{max} for lactose transport were 0.76 ± 0.07 mM and (1.45 ± 75) × 10^3 $nmol \cdot mg^{-1} \cdot min^{-1}$, respectively. The rates of lactose oxidation were corrected for background sGDH activity, using the rates of glucose oxidation and taking into account the small differences in V_{max} and K_m values of sGDH for lactose and glucose.

Finally, the transport rates in the proteoliposomes preloaded with 5 mM TMG were ≈ 1.5-fold lower when estimated by the spectroscopic assay than by the conventional method based on radiolabel distribution. This difference is most readily explained by the anomeric specificity of sGDH, as the enzyme only oxidizes the β-anomer rapidly, whereas both α-anomer and β-anomer are transported via LacS.

Transport activity in hybrid membranes

For the experiments described so far, purified and membrane-reconstituted LacS protein was used. The spectroscopic assay system is much more versatile when the protein purification and reconstitution steps can be eliminated, i.e. when membrane vesicles can be used. Membrane vesicles are easily obtained from both prokaryotic and eukaryotic cells. To investigate this possibility, the assay was applied to right-side-out membrane vesicles of *S. thermophilus* fused with liposomes. Both melibiose and lactose were used as substrate of the transporter [11]. Addition of hybrid membranes to assay buffer supplemented with 5 or 0.5 mM melibiose led to rapid oxidation of the sugar (Fig. 5). As observed with proteoliposomes, the oxidation was much faster for TMG-loaded than for unloaded hybrid membranes. Moreover, also in the hybrid membranes, the presence of 5 mM internal TMG eliminated the initial slow phase of oxidation, as was observed in the proteoliposomes (Fig. 5; compare traces a and b). The presence of a 10-fold excess of TMG on the outside completely abolished the transport of melibiose (Fig. 5; trace d), whereas a 10-fold excess of α-methylglucose had no effect (data not shown). Similar observations were made when lactose was used as substrate. This clearly indicates that the decrease in A_{600} results from LacS-mediated uptake of melibiose or lactose, followed by internal oxidation by sGDH. Although the K_m of sGDH for melibiose is much higher than for lactose, the transport activity is readily demonstrated and the background activity is only a small proportion of the transport activity. With lactose as substrate, an sGDH concentration of 50 $\mu g \cdot mL^{-1}$ was sufficient to make the transport step rate-determining. With melibiose, on the other hand, maximal activity was not yet attained at 100 $\mu g \cdot mL^{-1}$, which must reflect the low affinity of the enzyme for this substrate. Importantly, the background sGDH activity, caused by enzyme that remains associated with the outer surface of the membranes, is significantly lower in the hybrid membranes than in the proteoliposomes. The reason for this difference is not known, but is probably related to the fraction of endogenous lipids present in the hybrid membranes.

DISCUSSION

In this paper we describe a new spectroscopic assay for the characterization of carbohydrate-transport reactions. The assay is based on the oxidation of transported carbohydrates by an internal PQQ-dependent aldose dehydrogenase (sGDH) and the subsequent reduction of the electron acceptor Cl_2Ind. Because of the need for a well-characterized model system for the development and optimization of the assay, the lactose-transport protein (LacS) of *S. thermophilus* present in proteoliposomes or hybrid membranes was used in this study.

Although some oxidation of sugar by external sGDH was observed, the majority of the activity originated from transport into the vesicle lumen. The evidence for sugar oxidation after

uptake into the vesicle lumen is the following: (a) oxidation of lactose in proteoliposomes exceeds that in liposomes; (b) the kinetics of lactose oxidation in the proteoliposomes shows typical features of the LacS protein; (c) oxidation of lactose in proteoliposomes is inhibited by α-NPG; (d) similarly, the oxidation of lactose and melibiose in the hybrid membranes is inhibited by TMG and the aspecific activity is only a small percentage of the transport activity; (e) a *trans*-substrate, i.e. internal TMG, stimulates the oxidation of lactose (and melibiose) both in the proteoliposomes and hybrid membranes but not in the liposomes.

The multicomponent kinetics of lactose uptake in the proteoliposomes and hybrid membranes, i.e. an initial slow phase followed by a more rapid phase, is due to an increased contribution of exchange to the overall transport until a steady-state activity is reached. The slow phase would correspond to downhill transport of lactose in symport with an H^+. The rapid phase is assigned to the exchange mode of transport, in which external lactose is exchanged for internal lactono-δ-lactone. The homologous exchange of lactose has been shown to be one order of magnitude faster than the symport reaction [14]. Consistent with this hypothesis is the observation that TMG-loaded proteoliposomes and hybrid membranes showed more rapid oxidation of transported sugar without the initial slow phase. From the experiments with TMG-loaded proteoliposomes, it is obvious that, besides uniport/symport reactions, exchange reactions can also be studied with this assay, even though the transported sugar is oxidized.

It was important to establish whether or not the assay could be applied to a crude membrane system. This eliminates the need to purify the transporter and reinsert it in to the membrane, which makes the assay more generally applicable. Until now, transport studies in hybrid membranes were difficult to perform, because artificial ion gradients that drive the uphill uptake are transient in the relatively leaky hybrid membranes of Gram-positive bacteria. Moreover, uniport-type transport mechanisms, which are particularly prominent in eukaryotic sugar transporters, only allow equilibration of external and internal substrates, which is even more problematical to assay. Because in the spectroscopic assay the transport is driven by the concentration gradient which is maintained by the subsequent oxidation of the substrate, there is no requirement for a proton motive force or any other form of metabolic energy to accumulate the sugar.

The assay has some distinct advantages over assays based on label distribution: (a) there is no requirement for isotopically labelled substrate, which allows the use of a much wider range of commercially available substrates; (b) reactions can be followed directly 'on-screen', i.e. there is no delay between the performance of the experiments and the observation of the data; (c) data sampling is continuous and there is no step in which internal and external substrates are separated (both ensuring a high degree of accuracy); (d) transport systems with affinity constants for substrates in the millimolar range can be analyzed more accurately because high external substrate concentrations can be used; (e) transport reactions that do not require some form of metabolic energy, i.e. uniport reactions, can be followed with high sensitivity. As transport in the spectroscopic assay is driven by the concentration gradient which is maintained by the subsequent oxidation, it allows characterization of mutants in which the energy-coupling mechanism, that is the co-transport of H^+ or Na^+, is defective. This type of mutant is often encountered in site-directed mutagenesis studies, and they add to our understanding of energy coupling in transport [19,20]. The spectroscopic assay also provides a useful tool for characterizing facilitated diffusion via PTSs. For instance, in *Lactobacillus pentosus*, D-xylose is transported via the mannose-PTS without concomitant phosphorylation of the sugar [21]. It is one of our aims to determine whether or not a phosphorylated state of the membrane component(s) of the mannose-PTS is needed for the facilitated diffusion of xylose. Although the sugar uptake will always be downhill, the effects of an imposed membrane potential, pH or sodium gradient, on the translocation reaction can be studied. Finally, when an ATP-regenerating system can be incorporated together with sGDH in the proteoliposomes or hybrid membranes, the assay will even be applicable to ATP-driven carbohydrate-transport systems.

What are the limitations inherent in the spectroscopic assay of sugar transport? (a) Sugar accumulation levels cannot be determined, as the substrate is oxidized; (b) the system requires incorporation of sufficient sGDH to make the transport reaction rate-determining. This criterion, however, is readily met, as the enzyme has a very high turnover compared with transport systems (Fig. 4). The Michaelis–Menten kinetics of transport at the 'high' internal sGDH concentrations (Fig. 4) strongly indicate that lactose-oxidation rates reflect transport rates. Moreover, the values obtained from the spectroscopic assay compare well with those obtained from conventional assays of radiolabel distribution.

Despite these minor limitations, we feel that, in many instances, the assay can and will substitute for conventional assays of sugar transport, and thereby add to our understanding of various types of membrane transport. In principle, one could develop a similar assay system for the uptake of amino acids (or other substrates) by incorporating a suitable amino acid oxidase into the vesicle lumen. With the advent of genome-sequencing projects, numerous genes encoding putative transport systems from bacteria, archaea and eukarya have been identified. In many cases, the transport function is inferred from the homology of the proteins with known transport proteins, and often the system can be classified in terms of the nature of the substrate transported. The assay would be very useful for defining the substrate specificity of these newly identified transport proteins by screening a wide range of carbohydrates as putative substrates.

ACKNOWLEDGEMENTS

We would like to thank Professor J. A. Duine and A. Dewanti from the Technical University of Delft for kindly providing glucose dehydrogenase and PQQ. This work was supported by a grant from the EU (BIO4-CT96-0380).

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